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Long-term Phorbol Ester Treatment Down-regulates Protein Kinase C and Sensitizes the Phosphoinositide Signaling Pathway to Hormone and Growth Factor Stimulation

EVIDENCE FOR A ROLE OF PROTEIN KINASE C IN AGONIST-INDUCED DESENSITIZATION*

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Exposure of a nontransformed, continuous line of epithelial cells derived from rat liver (WB cells) to epidermal growth factor, angiotensin II, [Arg^8]vasopressin, and epinephrine resulted in rapid accumulation of the inositol phosphates (InsP) $InsP_1$, $InsP_2$, and $InsP_3$. Although short-term (5–60 min) pretreatment of WB cells with the phorbol ester 4β-phorbol 12β-myristate 13α-acetate (PMA) markedly attenuated InsP accumulation in response to all agonists, the inhibitory effects on the InsP response were lost after 2 h incubation with PMA; and, with extended (6–24 h) preincubation, a time-dependent potentiation of the InsP response to angiotensin II, epidermal growth factor and [Arg^8]vasopressin was observed. The InsP response during a 15-min challenge with angiotensin II in cells pretreated for 18 h with 600 nM and 10 μM PMA was increased by 2–3-fold and 4–6-fold, respectively. Long-term (18 h) treatment with 600 nM and 10 μM PMA caused a similar 90–100% loss of measurable Ca^{2+}/phospholipid-dependent enzyme (protein kinase C) activity in cytosolic and soluble particulate fractions. The effects of long-term PMA pretreatment do not represent a general enhancement of hormone responsiveness since the InsP response to epinephrine was not affected. In control cells, the InsP response to angiotensin II and epinephrine desensitized very rapidly. Long-term pretreatment with PMA greatly reduced the contribution of agonist-induced desensitization to the angiotensin II response; in contrast, the extent of desensitization occurring during incubation of WB cells with epinephrine was unaltered by long-term treatment with PMA suggesting that an additional mechanism may be involved in α_1-adrenergic receptor desensitization. No PMA-induced change in resting levels of [^{3}H]phosphoinositides or the metabolism of exogenous [^{3}H]inositol 1,4,5-trisphosphate by WB homogenates occurred. Stimulation of InsP formation in intact cells by NaF and activation of phospholipase C by GTPγS in membranes both were unaltered by short-term or long-term PMA pretreatment. These data are consistent with the idea that following long-term treatment of WB cells with PMA, the occurrence of agonist-induced desensitization of receptors linked to the phosphoinositide/Ca^{2+} signaling system is reduced, apparently at least in part due to the loss of contribution of a negative feedback regulatory role of protein kinase C.

Stimulation of a variety of cell surface receptors results in activation of phospholipase C and rapid hydrolysis of membrane $PtdIns(4,5)P_2$[1] to generate two second messengers, $Ins(1,4,5)P_3$ and diacylglycerol (DAG). $Ins(1,4,5)P_3$ releases Ca^{2+} from intracellular stores whereas DAG, in concert with membrane phospholipids and Ca^{2+}, promotes translocation to the plasma membrane and activation of Ca^{2+}/phospholipid-dependent enzyme (protein kinase C) (1–3). The tumor-promoting phorbol esters mimic the effects of endogenously produced DAG in activating protein kinase C (3) and, thus, have provided a powerful tool to study the role of protein kinase C in cell function. Short-term activation of protein kinase C with phorbol esters results in the inhibition of agonist-induced $PtdIns(4,5)P_2$ hydrolysis (2, 4–9) and Ca^{2+} mobilization (2, 7–10) in a variety of tissues. These observations suggest that protein kinase C activity may play an important role in the feedback regulation of the receptor-operated $Ins(1,4,5)P_3/Ca^{2+}$ signaling pathway.

The contribution of protein kinase C to cellular function also has been examined by the blockade of its activity. Several pharmacological agents (11) have been reported to inhibit protein kinase C activity with partial selectivity; and sphingosine, a major lipid component of the plasma membrane, has been shown to inhibit competitively the binding of phorbol esters to protein kinase C (12) and to inhibit protein kinase C-mediated processes (13, 14). Alternatively, chronic exposure of cells to phorbol esters results in down-regulation of protein kinase C and a concomitant loss of phorbol ester responsiveness (3, 15–18).

WB cells are a nontransformed, continuous line of epithelial cells derived from rat liver that have been shown previously

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[1] The abbreviations used are: $PtdIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PMA, 4β-phorbol 12β-myristate 13α-acetate; DAG, diacylglycerol; EGF, epidermal growth factor; InsP, total inositol phosphates including inositol monophosphate ($InsP_1$), inositol bisphosphate ($InsP_2$), inositol trisphosphate ($InsP_3$), and inositol tetrakisphosphate; $Ins(1,4)P_2$, inositol 1,4-bisphosphate; $Ins(1,3,4)P_3$, inositol 1,3,4-trisphosphate; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; PtdIns, phosphatidylinositol; PtdIns-4-P, phosphatidylinositol 4-phosphate; G-protein, guanine nucleotide regulatory protein; GTPγS, guanosine 5′-*O*-(3-thio)trisphosphate; HEPES, 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; Me_2SO, dimethyl sulfoxide.

to express functional EGF receptors (19). We have found that activation of EGF, angiotensin II, [Arg^8]vasopressin, and α_1-adrenergic receptors on WB cells results in the hydrolysis of phosphoinositides.[2] To investigate the role of protein kinase C activity in the regulation of agonist-stimulated PtdIns(4,5)P_2 hydrolysis and InsP formation in WB cells, we have characterized hormone- and growth factor-stimulated InsP formation under conditions where protein kinase C has been down-regulated by chronic exposure to the phorbol ester, PMA. We report that, although acute activation of protein kinase C by PMA results in a marked reduction in the accumulation of InsP to all agonists tested, down-regulation of protein kinase C reverses this effect of phorbol ester and results in a selective and marked increase in the levels of InsP formed to angiotensin II, [Arg^8]vasopressin, and EGF but, surprisingly, not to epinephrine. Studies designed to elucidate possible mechanisms underlying this sensitization of the phosphoinositide signaling pathway are described.

EXPERIMENTAL PROCEDURES

Materials—EGF was prepared from mouse maxillary glands as previously described (21). Angiotensin II and [Arg^8]vasopressin were obtained from Boehringer Mannheim; epinephrine, Me_2SO, BSA, and PMA were obtained from Sigma. *myo*-[^{3}H]Inositol (15 Ci/mol) was obtained from American Radiolabeled Chemical Co. (St. Louis, MO). WB cells were generously provided by Dr. J. W. Grisham, The University of North Carolina at Chapel Hill.

Cell Culture—WB cells were grown as previously described (19). WB cell stock cultures were maintained at 37 °C in an 8% CO_2, humidified atmosphere in Richter's Improved MEM containing insulin (4 mg/liter) and L-glutamine (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum and antibiotics. All stock plates were confluent and 5–7 days old at the time of subculture. Subculture was accomplished by aspirating the medium and detaching the cells by addition of 1 ml of trypsin (0.25%) in isotonic buffer for 30 min. Cells were seeded at a 1:5 dilution onto 100-mm plastic culture plates (Falcon), and the medium was replaced every 4th day. For most experiments, cells were routinely grown to confluency on 12-well plastic culture plates (Costar).

Analysis of Inositol Phosphates in Intact Cells—For all experiments, WB cells were grown until contact-inhibited and in a confluent state; cells were used 1 day postconfluency. WB cells were labeled for 18–24 h with 5–20 μCi/ml [^{3}H]inositol in inositol-free Dulbecco's modified Eagle's medium containing high glucose (4500 mg/liter) and supplemented with 5% fetal calf serum. When appropriate, PMA (10 mM stock) or vehicle (Me_2SO) was added directly to the labeling medium to a specified final concentration and for a specified time of pretreatment. At the end of the labeling period, the medium was replaced by Eagle's minimal essential medium-HEPES (25 mM) containing 10 mM LiCl, unless otherwise specified, and incubated for 10 min at 37 °C in the presence of room air. Agonists were prepared in the same medium (0.1% BSA final was added for EGF) and added for the indicated times, and the reactions were terminated by the addition of cold 5% perchloric acid. Samples were processed and analyzed for the presence of water-soluble [^{3}H]inositol phosphates by anion exchange chromatography as described previously (22) with modifications (23).

Determination of $InsP_3$ Isomers by HPLC—$InsP_3$ isomers were analyzed by HPLC using the method of Irvine *et al.* (24) and as described previously with minor modifications (25). ^{3}H-Labeled internal standards of Ins(1,3,4)P_3, Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4 were prepared and kindly supplied by Drs. Len Stephens, Phil Hawkins, and C. P. Downes, Smith Kline & French Research Ltd., Welwyn, England.

Measurement of Phosphoinositides—^{3}H-Labeled PtdIns, PtdIns-4-P, and PtdIns(4,5)P_2 were quantitated as described previously (26) employing modifications of the procedure described by Creba *et al.* (27).

Measurement of Inositol Phosphates in Membrane Preparations—Confluent cultures of WB cells in 100-mm dishes were labeled overnight with 10 μCi/ml [^{3}H]inositol as described above. To prepare WB membranes, the labeling medium was removed and replaced with cold hypoosmotic lysis buffer (1 mM HEPES, 1 mM EGTA, pH 7.0) and incubated at 4 °C for 20–30 min. The lysis buffer was removed by aspiration, the cells were scraped from the dishes with a rubber policeman, and the tissue was collected for centrifugation. The lysates were resuspended in approximately 8 ml of cold lysis buffer and pelleted twice by centrifugation at 15,000 × *g* for 10 min. The membrane pellet was resuspended in HEPES (10 mM, pH 7.0), pelleted by centrifugation, and resuspended in the same buffer for assay. Assay of phospholipase C activity and the formation of InsP in washed WB membranes to agonist and guanine nucleotides was as described previously by Harden *et al.* (26).

Measurement of Protein Kinase C Activity—Confluent cultures of WB cells were treated for 18 h with 0, 600 nM, or 10 μM PMA, homogenized, and then separated into cytosolic and particulate fractions by 105,000 × *g* centrifugation. The particulate fraction was extracted with 0.35% Triton, and then both the cytosolic and soluble particulate fractions were assayed for protein kinase C activity as described previously (28) by measuring the phosphatidylserine (63 and 600 μM)- and diacylglycerol (39 and 80 μM)-mediated transfer of ^{32}P from [γ-^{32}P]ATP to substrate, *i.e.* HPLC-purified *N*-bromosuccinimide cleavage fragment of lysine-rich histone (29).

Measurement of Ins(1,4,5)P_3 Metabolism in WB Homogenates—Confluent cultures of WB cells in 100-mm dishes were treated for 18 h with PMA or vehicle and lysed with hypoosmotic buffer as described above. Lysis buffer was removed by aspiration, the lysed cells were removed from the culture dishes with a rubber policeman, and the lysates were collected in a glass tube. Lysates were maintained at 4 °C and subjected to three consecutive 5-s bursts of sonication with a sonicator probe (Ystrom). Twenty μl of lysate was added to 80 μl of an intracellular buffer (26) containing [^{3}H]Ins(1,4,5)P_3 (1 Ci/mmol, Amersham) in a glass tube at 4 °C, vortexed, and incubated for the indicated times at 37 °C in the absence of LiCl. The assay was terminated by the addition of cold 5% perchloric acid and ^{3}H-labeled inositol phosphates were processed and resolved by Dowex chromatography as described above.

Statistical Analysis—Statistical analysis of the data was performed using a two-tailed Student's *t* test.

RESULTS

Stimulation of WB cells with maximally effective concentrations of EGF (300 ng/ml), epinephrine (10 μM), angiotensin II (1 μM), and [Arg^8]vasopressin (1 μM) resulted in a significantly enhanced accumulation of $InsP_1$, $InsP_2$, and $InsP_3$ as compared to unstimulated cells (Fig. 1). It has been reported previously that short-term pretreatment of cells with 0.1–1.0 μM PMA maximally activates protein kinase C and that long-term pretreatment with similar concentrations of PMA results in down-regulation of protein kinase C as assessed by measurable enzyme activity, immunoreactivity, and responsiveness to phorbol ester (3, 15–18). As illustrated in Table I, pretreatment of WB cells with 600 nM PMA for 15 min resulted in an 85–100% loss of response to agonists. In contrast, overnight (18 h) pretreatment of WB cells with 600 nM PMA resulted in markedly different changes in hormone responsiveness. The InsP response to epinephrine was similar to that observed with control cells, but InsP accumulation in response to angiotensin II and EGF were enhanced significantly over control responses (Table I). As illustrated in Fig. 2, PMA-induced potentiation of agonist-stimulated formation of InsP occurred without a noticeable change in the $K_{0.5}$ for either agonist (*i.e* 30 ng/ml and 1 nM for EGF and angiotensin II, respectively). Incubation of WB cells with 10 μM of the inactive analogue of PMA, 4α-phorbol, for 15 min or 18 h had no effect on hormone responsiveness (data not shown).

A 15-min pretreatment of WB cells with various concentrations of PMA resulted in a concentration-dependent loss of response to angiotensin II; maximal inhibition (85%) of hormone responsiveness was observed with 1–10 μM PMA (Fig. 3). Similarly, 15-min pretreatment with 10 μM PMA completely blocked EGF-, epinephrine-, and [Arg^8]vasopressin-

[2] Earp, H. S., Hepler, J. R., Petch, L. A., Miller, A., Raymond, V. W., McCune, B. K., Lee, L. W., Grisham, J. W., and Harden, T. K., submitted for publication.

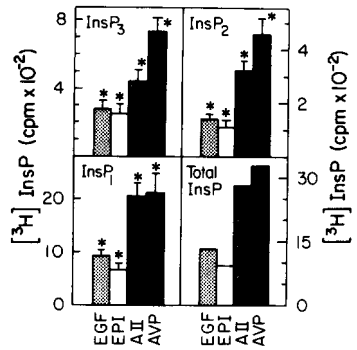

FIG. 1. **Hormone- and growth factor-stimulated accumulation of inositol phosphates in WB cells.** WB cells were labeled overnight with 20 μCi/ml [^{3}H]inositol and then treated with vehicle (0.1% BSA), 300 μM epidermal growth factor (*EGF, stippled*), 10 μM epinephrine (*EPI, white*), 1 μM angiotensin II (*AII, hatched*), or 1 μM [Arg^8]vasopressin (*AVP, black*) for 5 min in the presence of 10 mM LiCl. Samples were assayed for [^{3}H]inositol phosphates by anion exchange chromatography. The data are expressed as the increase in ^{3}H radioactivity over vehicle. The levels of ^{3}H label measured in the presence of vehicle for $InsP_1$, $InsP_2$, and $InsP_3$ were 1083 ± 105, 196 ± 3, and 576 ± 23, respectively, with most of this radioactivity (see figure legends below) representing counts/min present in the $T = 0$ blank sample. The values given are the mean ± S.E. of quadruplicate determinations and are representative of three experiments. *, the increase in inositol phosphate formation due to agonist stimulation was significantly greater than vehicle control ($p < 0.05$).

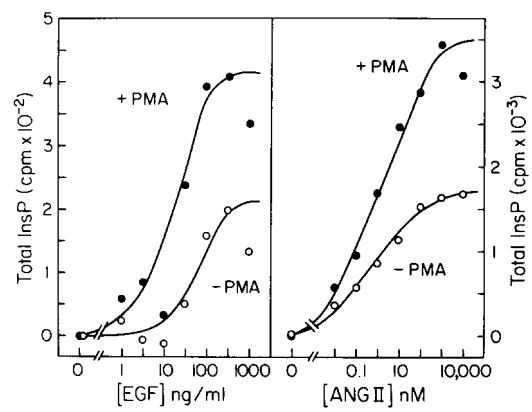

FIG. 2. **PMA-induced potentiation of epidermal growth factor- and angiotensin II (*ANG II*)-stimulated inositol phosphate formation in WB cells.** WB cells were labeled for 18 h with 4 μCi/ml [^{3}H]inositol in the presence of 0.01% Me_2SO (−PMA; ○) or 600 nM PMA (+PMA; ●) and then treated with epidermal growth factor (*left panel*), or angiotensin II (*right panel*) at the indicated concentrations for 15 min in the presence of 10 mM LiCl. Samples were assayed for total [^{3}H]inositol phosphates by anion exchange chromatography. The values of radioactivity accumulated in the presence of vehicle were 579 ± 12 and 546 ± 14 cpm for control and PMA-pretreated cells, respectively, and were subtracted from the values presented. The data are presented as the mean of triplicate determinations and are representative of two experiments.

TABLE I

Differential effects of short-term or long-term PMA pretreatment on agonist-stimulated inositol phosphate accumulation in WB cells

WB cells were labeled overnight with 4 μCi/ml [^{3}H]inositol and pretreated for the final 15 min or 18 h of this labeling period with 0.01% Me_2SO or 600 nM PMA. Cells were then challenged with vehicle (0.1% BSA), or the indicated agonists for 15 min. Samples were assayed for [^{3}H]inositol phosphates by anion exchange chromatography. Initial resting levels of radioactivity were subtracted from the values presented. The data are the mean ± S.E. of quadruplicate determinations and are representative of four experiments.

Treatment	[^{3}H]Inositol phosphate accumulation		
	Control	+PMA (600 nM)	
		15-min pretreat	18-h pretreat
	cpm ± S.E.	*cpm ± S.E.*	*cpm ± S.E.*
Vehicle	23 ± 45	83 ± 47	128 ± 85
EGF (300 ng/ml)	354 ± 64	35 ± 58[a]	629 ± 53[b]
Epinephrine (10 μM)	587 ± 47	58 ± 111[a]	733 ± 55
Angiotensin II (1 μM)	1407 ± 163	253 ± 116[a]	3547 ± 238[b]

[a] The increase in inositol phosphate formation to agonist in PMA-treated cells is significantly less than that in control cells ($p < 0.05$).

[b] The increase in inositol phosphate formation to agonist in PMA-treated cells is significantly greater than that in control cells ($p < 0.05$).

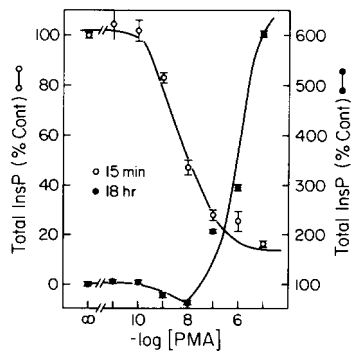

FIG. 3. **Differential modification of angiotensin II-stimulated inositol phosphate formation in WB cells after short-term or long-term pretreatment with PMA.** WB cells were labeled overnight with 5 μCi/ml [^{3}H]inositol and pretreated for the final 18 h (●) or 15 min (○) of this labeling period with 0.01% Me_2SO or the indicated concentrations of PMA. Cells were then challenged with vehicle or 1 μM angiotensin II for 15 min. Samples were assayed for [^{3}H]inositol phosphates by anion exchange chromatography. Angiotensin II-stimulated increases in inositol phosphates in control cells were 1124 ± 8 and 1108 ± 12 cpm for 18-h and 15-min experiments, respectively, and were defined as 100% response. Each value is the mean ± S.E. of triplicate determinations.

stimulated InsP formation (data not shown). The changes observed after long-term treatment with PMA also were dependent on the concentration of PMA. That is, lower concentrations of PMA (0.1–10 nM) resulted in attenuation of hormone responsiveness, whereas a marked concentration-dependent potentiation of the agonist-induced InsP response occurred with higher concentrations of phorbol ester (0.1–10 μM; Fig. 3). Under these conditions, pretreatment with 10 μM PMA resulted in a 6-fold increase in angiotensin II-stimulated InsP response measured over a 15-min period. No change in the light microscopic appearance of confluent, contact-inhibited cells was observed after 18 h with 10 μM PMA. Long-term pretreatment of cells with 10 μM PMA also resulted in a marked increase in the EGF and [Arg^8]vasopressin InsP responses (data not shown) but did not alter the epinephrine-mediated accumulation of InsP (see below).

As shown in Fig. 4, the time course for modulation of the InsP response by PMA (10 μM) was clearly biphasic. Brief pretreatment inhibited the InsP response to a maximally effective concentration (1 μM) of angiotensin II. An 85% blockade of the response was observed within the first 5 min, and loss of response was maintained for up to 60 min (Fig. 4). Within 2 h, the inhibitory effects of PMA on the InsP response were lost, and with extended preincubation times a time-dependent potentiation of the InsP response occurred. After 10 h of pretreatment, the levels of InsP formed in response to a 10-min challenge with agonist were potentiated by approximately 4-fold (Fig. 4). This potentiation was maintained for at least 24 h in the continued presence of PMA.

Since long-term pretreatment of cells with phorbol ester

has been reported to down-regulate protein kinase C (3, 15–18), the activity of protein kinase C was measured directly in cytosolic and particulate fractions prepared from WB cells pretreated for 18 h with PMA. As shown in Table II, pretreatment of WB cells with 600 nM and 10 μM PMA resulted in a 90–100% loss of measurable protein kinase C activity as assessed by phosphatidylserine/diacylglycerol-induced ^{32}P labeling of substrate (HPLC-purified *N*-bromosuccinimide cleavage fragment of lysine-rich histone).

A time course for agonist-induced InsP formation of WB cells revealed that maximally effective concentrations of angiotensin II and epinephrine each stimulated a rapid accumulation of InsP. However, accumulation of InsP in response to both agonists was essentially maximal within 60 s (Fig. 5). Chronic pretreatment (18 h) with 10 μM PMA produced markedly different effects on response to the two agonists. Whereas PMA pretreatment had no effect on the time course of accumulation of InsP in the presence of epinephrine (Fig. 5, *right*), phorbol ester pretreatment markedly sensitized the inositol phosphate pathway to angiotensin II stimulation (Fig. 5, *left*). Thus the "fold stimulation" produced by angiotensin II was 4-, 6-, and 9-fold after a 10-, 15-, and 20-min stimulation, respectively. Under these conditions, basal accumulation of InsP was not augmented by long-term pretreatment with PMA. A more detailed time course for the initial 90 s of the angiotensin II-stimulated InsP response in PMA-pretreated *versus* control cells is presented in Fig. 6. Angiotensin II-stimulated InsP accumulation was maximal within 30 s after hormone addition to control cells. In contrast, the accumulation of InsP in PMA-pretreated cells was greater than that observed in control cells, and after 30 s InsP continued to accumulate, although at a slower rate than initially.

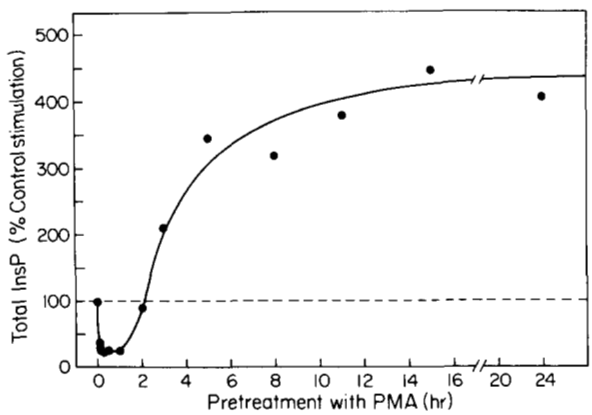

FIG. 4. **Time course for PMA-induced modification of the angiotensin II-stimulated accumulation of inositol phosphates.** WB cells were labeled overnight with 5 μCi/ml of [^{3}H]inositol in the presence of 10 μM PMA or 0.01% Me_2SO for the indicated times. Following preincubation, cells were challenged with 1 μM angiotensin II or vehicle in the presence of 10 mM LiCl for 10 min and assayed for the accumulation of [^{3}H]inositol phosphates. At each time point, angiotensin II stimulation after PMA pretreatment was compared to angiotensin II stimulation after Me_2SO treatment. The fold stimulation due to angiotensin II over vehicle was arbitrarily defined as 100% maximal stimulation for each time. Each value is the mean of triplicate determinations and the data are representative of two experiments.

One interpretation of data presented thus far is that, following long-term pretreatment of WB cells with PMA, angiotensin II (and other) receptor-mediated stimulation of phospholipase C does not result in desensitization of the receptor/phospholipase C response system. Experiments were carried out to demonstrate more directly that this was the case (Fig. 7). Control and PMA-pretreated (10 μM, 18 h) cells were incubated with 1 μM angiotensin II for 30 min in the absence of LiCl. The cells were then rapidly washed free of agonist and fresh medium was added containing angiotensin II (1 μM) and LiCl (10 mM). Since LiCl inhibits $InsP_1$ metabolism (2), it was reasoned that any phosphoinositide hydrolysis still occurring in the angiotensin II-pretreated cells would be detected as an increase in total InsP after rechallenge with angiotensin II plus LiCl. In contrast to the situation in control cells where readdition of angiotensin II and LiCl elicited only a very small increase in InsP, readdition of this hormone plus LiCl to PMA-pretreated cells resulted in a large accumulation of InsP which was maintained at a linear rate for at least 30 min (Fig. 7, 30-min time point not shown). The data suggest that the difference in hormone responsiveness between con-

TABLE II

Protein kinase C activity after long-term PMA treatment

WB cells were maintained for 18 h in the absence or presence of PMA. Homogenates were then prepared and protein kinase C activity, *i.e.* phosphatidylserine + diacylglycerol (PS/D)-induced [^{32}P]phosphorylation of substrate, was determined as described under "Experimental Procedures." The data are the mean results of three and two separate experiments for the low PS/D and high PS/D, respectively, and each experiment consisted of triplicate determinations. Blank levels of ^{32}P radioactivity (PS/D-induced phosphorylation in the absence of substrate) were subtracted from the values presented.

Kinase activity	Protein kinase C activity					
	Cytosolic			Soluble particulate		
	Control	+PMA 600 nM	+PMA 10 μM	Control	+PMA 600 nM	+PMA 10 μM
	[^{32}P] cpm					
a. Low phosphatidylserine (63 μM) and diacylglycerol (39 μM)						
Basal	614	816	576	2460	2036	1508
Phosphatidylserine + diacylglycerol	2285	980	654	2820	2019	1312
Protein kinase activity	1671	164	78	360	−17	−196
% Control protein kinase C activity	100%	9.8%	4.6%	100%	0%	0%
b. High phosphatidylserine (600 μM) and diacylglycerol (80 μM)						
Basal	407	583	607	3359	2470	2664
Phosphatidylserine + diacylglycerol	4190	633	657	3955	2025	1740
Protein kinase C activity	3783	50	50	596	−445	−924
% Control protein kinase C activity	100%	1.3%	1.3%	100%	0%	0%

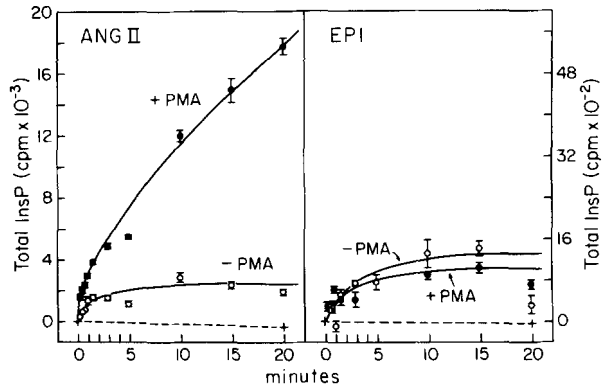

FIG. 5. **Differential effects of long-term PMA pretreatment on the time course of angiotensin II (*ANG II*)- and epinephrine-stimulated inositol phosphate accumulation in WB cells.** WB cells were labeled for 18 h with 5 μCi/ml [^{3}H]inositol in the presence of 0.01% Me_2SO (○) or 10 μM PMA (●) and then challenged with vehicle (+), 1 μM angiotensin II (*ANG II, left panel*), or 10 μM epinephrine (*EPI, right panel*) in the presence of 10 mM LiCl for the indicated times. Samples were assayed for the accumulation of [^{3}H] inositol phosphates by anion exchange chromatography. Initial (T = 0) resting levels of radioactivity in PMA- and Me_2SO-pretreated cells were 2681 ± 129 and 2260 ± 86, respectively, and were subtracted from the values presented. The data are the mean ± S.E. of triplicate determinations and are representative of three experiments.

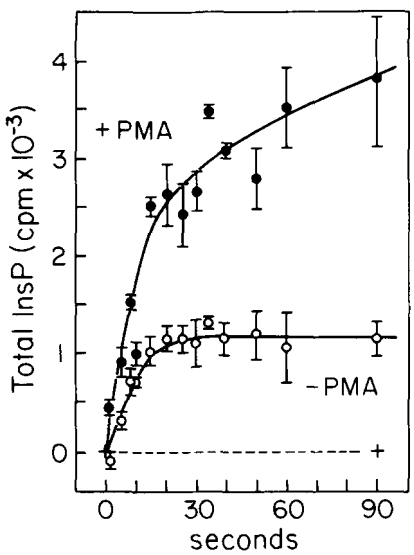

FIG. 6. **Potentiation of angiotensin II-stimulated inositol phosphate accumulation after long-term PMA pretreatment.** WB cells were labeled for 18 h with 5 μCi/ml [^{3}H]inositol in the presence of 0.01% Me_2SO (○) or 10 μM PMA (●) and then challenged with 1 μM angiotensin II (○, ●) or vehicle (+) for the indicated times. Samples were assayed for the accumulation of [^{3}H]inositol phosphates by anion exchange chromatography. Initial (T = 0) resting levels of radioactivity in PMA- and Me_2SO-pretreated cells were 3196 ± 76 and 3586 ± 107, respectively, and were subtracted from the values presented. The data are the mean ± S.E. of triplicate determinations.

trol and long-term PMA-pretreated WB cells can be explained at least in part by a reduction in the extent of agonist-induced desensitization in the PMA-pretreated cells.

Since long-term PMA pretreatment influenced the relative rate of formation of InsP to agonists, it is possible that substrate, *i.e.* phosphoinositide, levels and/or the specificity of phospholipase C for the different lipids was altered. To investigate these possibilities, we measured angiotensin II-induced changes over time in the levels of the phosphoinositides PtdIns, PtdIns-4-P, and PtdIns(4,5)P_2 and the individ-

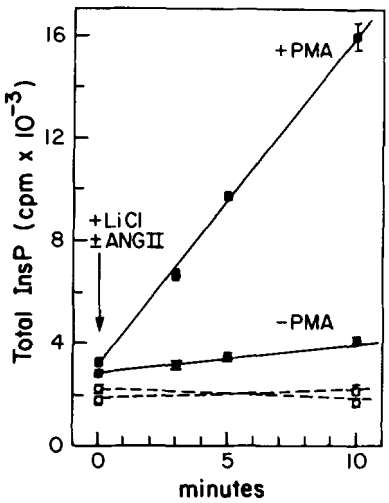

FIG. 7. **Blockade of angiotensin II-induced desensitization of the inositol phosphate response in WB cells by long-term PMA pretreatment.** WB cells were pretreated for 18 h with [^{3}H] inositol in the presence of 0.01% Me_2SO (○, ●) or 10 μM PMA (□, ■) and then challenged with 1 μM angiotensin II (●, ■) or vehicle (○, □) for 30 min in the absence of LiCl. The medium was rapidly aspirated, and the cells were rechallenged at T = 0 with fresh medium containing 1 μM angiotensin II (●, ■) or vehicle (○, □) in the presence of 10 mM LiCl. Samples were collected at the indicated times and were assayed for the accumulation of total [^{3}H]inositol phosphates by anion exchange chromatography. The data are the mean ± S.E. of triplicate determinations and are representative of three experiments.

ual inositol phosphates (Fig. 8). Chronic pretreatment of cells with PMA did not increase [^{3}H]inositol labeling of phospholipids since initial levels of radioactivity associated with each of the phosphoinositides were essentially the same in PMA pretreated and control cells (see legend, Fig. 8). In control cells, angiotensin II stimulated a rapid, albeit transient, 20 and 25% decrease in radioactivity associated with PtdIns(4,5)P_2 and PtdIns-4-P levels, respectively, over the initial 60 s (Fig. 8, *left*); lipid levels returned to initial values within 5 min. Significant changes in PtdIns levels were not observed. In contrast, a more rapid and pronounced 70 and 50% reduction in radioactivity associated with PtdIns(4,5)P_2 and PtdIns-4-P levels, respectively, occurred within the first 30 s of challenge of PMA-pretreated cells with angiotensin II (Fig. 8, *left*). Lipid levels gradually returned to initial levels over a 10-min period. As with control cells, significant changes in PtdIns levels were not observed. The relative ratios of the levels of radioactivity associated with PtdIns, PtdIns-4-P, and PtdIns(4,5)P_2 were 100:5.4:4.3, respectively, in control cells and 100:5.2:4.0, respectively, in PMA-pretreated cells.

Consistent with results obtained by measuring total InsP levels, angiotensin II-stimulated accumulation of InsP, $InsP_2$, and $InsP_3$ was potentiated in PMA-pretreated cells (Fig. 8, *right*). In control cells, agonist stimulated a rapid although transient rise in $InsP_3$ and $InsP_2$ levels; maximal levels for both inositol phosphates were obtained within 60 s. Elevated levels returned to base line within 5 min. $InsP_1$ levels were unchanged over the 1st min, and then a linear accumulation was observed for up to 20 min. PMA pretreatment resulted in a pronounced increase in the rate of accumulation of all three InsP (Fig. 8, *right*). $InsP_1$ levels were enhanced slightly over the 1st min and accumulated linearly at an accelerated rate in PMA-treated cells as compared to control cells for up to 20 min (Fig. 8, *right*); angiotensin II stimulated a rapid rise in the levels of both $InsP_2$ and $InsP_3$ in the 1st min which continued after 1 min at a slower rate for up to 20 min. Measurement of the two isomers of $InsP_3$, Ins(1,4,5)P_3 and

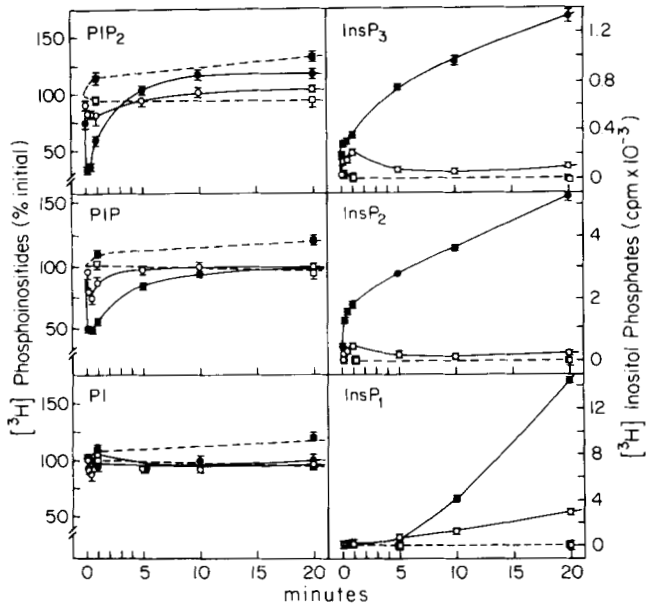

FIG. 8. **Potentiation of angiotensin II-stimulated turnover of phosphoinositides and formation of inositol phosphates in WB cells by long-term pretreatment with PMA.** WB cells were treated for 18 h with 5 μCi/ml [^{3}H]inositol in the presence of 0.01% Me_2SO (○, ■) or 10 μM PMA (○, ■) and then challenged with 1 μM angiotensin II (○, ●) or vehicle (□, ■) for the indicated times. Samples were assayed for changes in levels of the ^{3}H-labeled phosphoinositides, PtdIns(4,5)P_2 (*PIP$_2$, left top*), PtdIns-4-P (*PIP, left middle*), and PtdIns (*PI, left bottom*), and for accumulation of the ^{3}H-labeled inositol phosphates, $InsP_3$ (*right top*), $InsP_2$ (*right middle*), and $InsP_1$ (*right bottom*) as described under "Experimental Procedures." Angiotensin II-stimulated changes in the levels of each individual phosphoinositide were expressed as a percentage of initial ($T = 0$) resting levels, defined as 100%. Initial levels of ^{3}H radioactivity in PtdIns(4,5)P_2, PtdIns-4-P, and PtdIns were 1,892 ± 30, 2,421 ± 128, and 44,379 ± 2,255 cpm, respectively, in Me_2SO-treated cells, and 1,532 ± 70, 2,031 ± 15 and 38,683 ± 71 cpm, respectively, in PMA-treated cells. Background ($T = 0$) levels for $InsP_3$, $InsP_2$, and InsP were 171 ± 8, 220 ± 17, and 2,150 ± 121 cpm, respectively, in Me_2SO-treated cells, and 179 ± 5, 233 ± 19, and 2,781 ± 40 cpm, respectively, in PMA-treated cells and were subtracted from the values presented. The data are the mean ± S.E. for three determinations.

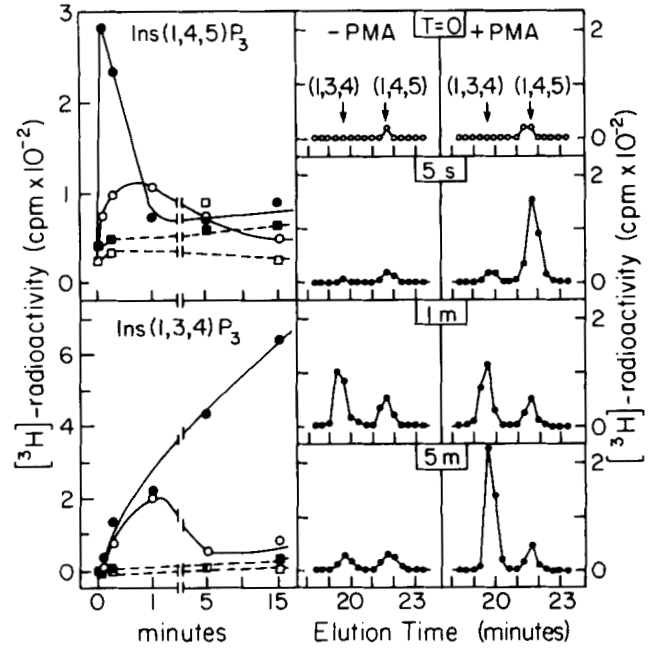

FIG. 9. **PMA-induced potentiation of angiotensin II-stimulated accumulation of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 in WB cells.** *Left side*, WB cells were labeled for 18 h with 10 μCi/ml [^{3}H] inositol in the presence of 0.01% Me_2SO (○, □) or 10 μM PMA (●, ■) and then challenged with vehicle (□, ■) or angiotensin II (○, ●) in the presence of 10 mM LiCl for the indicated times. Samples were assayed for the accumulation of $InsP_3$ isomers by HPLC as described under "Experimental Procedures." The data are single determinations and are representative of two experiments. *Right side*, HPLC elution profile of [^{3}H]$InsP_3$ isomers isolated from WB cells pretreated with 0.01% Me_2SO or 10 μM PMA for 18 h. Elution profiles depict ^{3}H radioactivity that coeluted with standards of Ins(1,3,4)P_3 and Ins(1,4,5)P_3, indicated by the *arrows*, in unstimulated WB cells exposed to 10 mM LiCl ($T = 0$, *top*) or in cells stimulated with 1 μM angiotensin II for 5 s, 1 min, or 5 min in the presence of 10 mM LiCl.

Ins(1,3,4)P_3, during stimulation with angiotensin II revealed that long-term pretreatment of the cells with PMA results in an increase in the initial rate of accumulation of Ins(1,4,5)P_3 as compared to control cells (Fig. 9). After the 1st min of incubation with angiotensin II, the levels of Ins(1,4,5)P_3 fell to a new steady state, reflecting a sustained, enhanced rate of formation. Further evidence for this was observed by the enhanced rate of accumulation of Ins(1,3,4)P_3, a metabolic product of Ins(1,4,5)P_3 in PMA-treated cells. Unlike those of Ins(1,4,5)P_3, the levels of Ins(1,3,4)P_3 did not establish steady state but rather continued to rise over a 15-min period (Fig. 9), perhaps due in part to the presence of 10 mM LiCl, an inhibitor of the 1-phosphomonoesterase responsible for metabolism of Ins(1,3,4)P_3 (30).

It has been reported that activation of protein kinase C by phorbol esters results in the phosphorylation and activation of a 5′-phosphomonoesterase resulting in an increased rate of degradation of inositol phosphates (31, 32). To test if the observed increase in the hormone-stimulated levels of InsP after chronic pretreatment of WB cells with phorbol ester was a partial consequence of an altered rate of degradation of InsP, we measured the rate of disappearance of exogenously added [^{3}H]Ins(1,4,5)P_3 and the rate of appearance of $InsP_2$ and $InsP_1$ in homogenates prepared from control and PMA-pretreated WB cells (Fig. 10). PMA pretreatment and down-regulation of protein kinase C did not alter the rate of degradation Ins(1,4,5)P_3 or the rate of formation of $InsP_2$ and

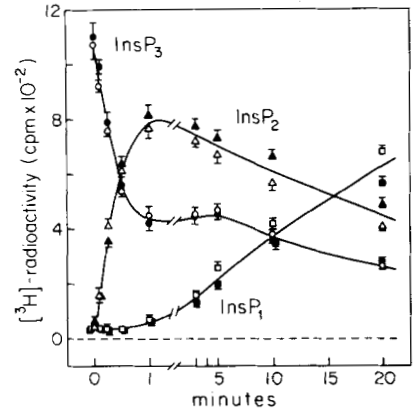

FIG. 10. **Lack of effect of long-term PMA pretreatment on the rate of degradation of $InsP_3$ in WB homogenates.** WB cells were pretreated for 18 h with 0.01% Me_2SO (*open symbols*) or 10 μM PMA (*closed symbols*), rapidly lysed, and prepared as homogenates as described under "Experimental Procedures." The homogenates were incubated in the presence of intracellular buffer and exogenously added [^{3}H]Ins(1,4,5)P_3 at 37 °C for the indicated times, and samples were assayed for the presence of ^{3}H radioactivity in $InsP_3$ (○, ●), $InsP_2$ (△, ▲), and $InsP_1$ (□, ■) by anion exchange chromatography. The data are expressed as the mean ± S.E. of triplicate determinations and are representative of two experiments.

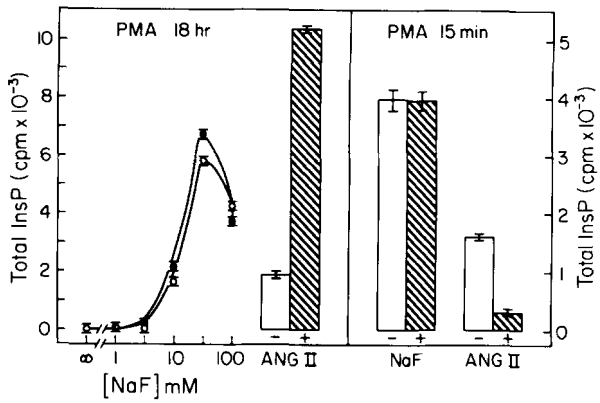

FIG. 11. **Differential effects of PMA pretreatment on inositol phosphate formation by NaF and angiotensin II in intact WB cells.** WB cells were labeled overnight with 5 μCi/ml [^{3}H] inositol. *Left side*, WB cells were pretreated for the final 18 h of the labeling period with 0.01% Me_2SO (*open circles*; −, *white bar*) or 10 μM PMA (*closed circle*; +, *hatched bar*), challenged with vehicle, NaF (*circles*) at the indicated concentrations, or 1 μM angiotensin II (*ANG II, bars*) in the presence of 10 mM LiCl for 15 min, and then assayed for total [^{3}H]inositol phosphates by anion exchange chromatography. *Right side*, WB cells were pretreated for the final 15 min of the labeling period with 0.01% Me_2SO (−, *white bars*) or 10 μM PMA (+, *hatched bars*), challenged with vehicle, 30 mM NaF, or 1 μM angiotensin II for 15 min, and then assayed for total [^{3}H]inositol phosphates by anion exchange chromatography. Levels of radioactivity accumulated in the presence of vehicle were subtracted from each value presented. The data are the mean ± S.E. of triplicate determinations and are representative of two experiments.

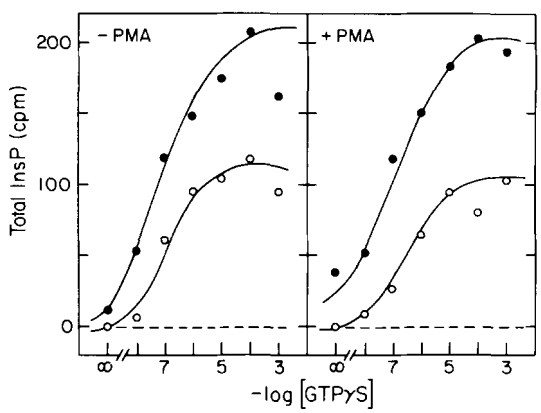

FIG. 12. **Stimulation of inositol phosphate formation by guanine nucleotide and angiotensin II in washed membranes prepared from control and PMA-pretreated cells.** WB cells were preincubated with 10 μCi/ml [^{3}H]inositol in the presence of 0.01% Me_2SO (*left panel*) or 10 μM PMA (*right panel*) for 18 h and prepared as washed membranes as described under "Experimental Procedures." Membranes were incubated at 37 °C for 15 min in assay buffer containing the indicated concentrations of GTPγS in the absence (○) or presence (●) of angiotensin II, and samples were assayed for total [^{3}H]inositol phosphates by anion exchange chromatography. The data are the mean of triplicate determinations and represent pooled results from two separate experiments.

$InsP_1$ in WB homogenates.

Hormone-stimulated activation of phospholipase C involves a G-protein (26, 33–41), and thus NaF, a well known activator of G-proteins, stimulates InsP formation in membrane preparations (36, 38, 39) and in intact cells (40, 41). To determine if chronic PMA pretreatment modified G-protein-stimulated InsP formation, we measured levels of InsP in intact control and PMA-treated WB cells challenged with NaF. As shown in Fig. 11 (*left*), NaF stimulated the accumulation of InsP in a concentration-dependent manner. Chronic pretreatment of the cells with PMA resulted in a marked potentiation of the angiotensin II-stimulated InsP response but was without effect on the NaF-stimulated InsP response (Fig. 11, *left*). Similarly, acute activation of protein kinase C by PMA inhibited the angiotensin II-stimulated InsP response by 85% but did not alter the levels of InsP formed in response to NaF stimulation (Fig. 11, *right*).

To test further the role of protein kinase C in regulation of agonist and G-protein-mediated InsP formation, we measured the formation of InsP in response to the nonhydrolyzable analogue of GTP, GTPγS, in the presence and absence of angiotensin II in washed membranes prepared from WB cells (Fig. 12). In control cells GTPγS stimulated InsP formation in a concentration-dependent fashion with a $K_{0.5}$ of approximately 3 μM (Fig. 12, *left*). A maximally effective concentration of angiotensin II, although ineffective alone, potentiated the InsP response to guanine nucleotide at all concentrations (Fig. 12, *left*). Chronic pretreatment with phorbol ester did not alter the levels of InsP accumulated in response to guanine nucleotide or agonist plus guanine nucleotide (Fig. 12, *right*). Short-term (20 min) pretreatment of WB cells with 10 μM PMA also did not alter the levels of InsP formed in response to GTPγS in washed membrane preparations (data not shown).

DISCUSSION

Hormone receptor-stimulated PtdIns(4,5)P_2 hydrolysis, InsP formation, and the subsequent rise in cytosolic Ca^{2+} can be abolished by short-term activation of protein kinase C (2, 4–10). These observations suggest that protein kinase C may play a regulatory role in receptor-stimulated PtdIns(4,5)P_2 hydrolysis. One prediction inherent in this idea would be that blockade or loss of protein kinase C activity would result in an increased responsiveness of the phosphoinositide signaling pathway to hormone stimulation. To address this question, we have characterized hormone- and growth factor-stimulated InsP formation under conditions where protein kinase C has been down-regulated by long-term exposure to the phorbol ester, PMA.

The present findings demonstrate that long-term pretreatment of WB cells with PMA results in a marked sensitization of the phosphoinositide signaling pathway to hormone and growth factor stimulation. This observed effect of phorbol ester pretreatment could occur by at least two possible means: (*a*) some component(s) in the phosphoinositide signaling pathway could be modified resulting in a system that is inherently more responsive to hormone stimulation, or (*b*) agonist-induced desensitization of the phosphoinositide signaling pathway may no longer occur due to down-regulation of protein kinase C and the loss of protein kinase C-mediated negative feedback inhibition. The present data are consistent with the latter idea. Kinetic analysis indicated that hormone-induced desensitization of the phosphoinositide signaling system occurs rapidly in WB cells. Desensitization may occur by more than one mechanism in these cells, but several lines of evidence suggest that responsiveness is regulated at least in part through activation of protein kinase C with consequent feedback inhibition of responsiveness. Consistent with this conclusion from kinetic analyses, short-term activation of protein kinase C with phorbol ester results in a marked inhibition of hormone-stimulated InsP formation in WB cells and many other tissues (2, 4–10). Additionally, sensitization of the phosphoinositide pathway to hormone stimulation in WB cells after long-term pretreatment with phorbol ester is correlated with a loss of measurable protein kinase C activity and a concomitant loss of phorbol ester responsiveness.

The conclusion that the increased hormone responsiveness occurring after down-regulation of protein kinase C is at least in part explained by a lack of protein kinase C-mediated agonist-induced desensitization upon hormone challenge is supported by several other observations. First, it has been reported in several tissues that activation of protein kinase C by phorbol ester results in phosphorylation and activation of a 5′-phosphomonoesterase responsible for the conversion of $Ins(1,4,5)P_3$ to $Ins(1,4)P_2$ (31, 32). This does not appear to be a major contributing factor in WB cells since the altered kinetics of InsP accumulation could not be explained by a simple change in the rate of metabolism of $Ins(1,4,5)P_3$ and direct analysis of metabolism of inositol phosphates in WB cell lysates failed to reveal any major PMA-induced alteration. Second, short-term phorbol ester pretreatment of certain cells stimulates an increased rate of labeling of $PtdIns(4,5)P_2$ (42, 43) and an increase in the levels of InsP formed to subsequent hormone stimulation (43). However, down-regulation of protein kinase C in WB cells did not alter either the amount of radioactivity associated with phosphoinositides or the relative distribution of label among the three phosphoinositides.

Receptor-mediated activation of phospholipase C involves a yet-to-be identified G-protein (33–39). At least one well characterized G-protein has been shown to be phosphorylated by protein kinase C (44) and it is possible that protein kinase C could alter hormone-stimulated InsP formation by inhibiting the action of the involved G-protein. Consistent with this idea are the recent observations that hormone- and guanine nucleotide-stimulated InsP formation in washed membranes prepared from 1321N1 astrocytoma cells (45) and polymorphonuclear leukocytes (46) after short-term pretreatment with phorbol ester was partially attenuated as compared to control membranes. This effect of phorbol ester in astrocytoma cells was mimicked to a limited extent by the addition of purified protein kinase C (45). The present results suggest that in WB cells, protein kinase C does not regulate agonist-stimulated $PtdIns(4,5)P_2$ hydrolysis by altering the capacity of the involved G-protein to active phospholipase C. That is, guanine nucleotide-stimulated InsP formation in washed membranes prepared from WB cells after short-term or long-term pretreatment with PMA was not significantly different from control. Furthermore, NaF-stimulated formation of InsP in intact cells remained unchanged under conditions where the hormone-stimulated response was markedly altered either by short-term or long-term PMA pretreatment. In contrast to the change in response observed with intact cells, InsP formation in the presence of guanine nucleotide plus angiotensin II was the same in washed membranes prepared from control cells as in membranes from protein kinase C down-regulated cells. Since greater than 80% of the measurable protein kinase C activity in unstimulated WB cells is cytosolic (Table II), a washed membrane preparation from untreated cells would contain a very limited amount of protein kinase C. Clearly, other cytosolic factors that are critical for the regulation of hormone-stimulated $PtdIns(4,5)P_2$ hydrolysis also could be lost in a washed membrane preparation.

Acute pretreatment of Swiss 3T3 cells with phorbol ester results in inhibition of bombesin-stimulated InsP formation but not platelet-derived growth factor-stimulated effects (47). Similarly, acute activation of protein kinase C by phorbol ester in primary cultures of rat hepatocytes selectively inhibits the α_1-adrenergic receptor-mediated InsP formation but leaves the vasopressin- and the angiotensin II-stimulated responses relatively unimpaired (48, 49). Although this finding is in contrast to the present results where short-term exposure of WB cells to phorbol ester inhibited α_1-adrenergic, angiotensin II-, and [Arg^8]vasopressin-stimulated InsP formation, these reports illustrate that phorbol esters inhibit agonist-stimulated InsP formation in a receptor- and tissue-selective manner. Such selectivity of action is consistent with the idea that protein kinase C acts at the level of the receptor rather than at a common point of convergence in the pathway such as a G-protein or the effector enzyme, phospholipase C. The present results also provide evidence in support of this idea. First, agonist-stimulated $PtdIns(4,5)P_2$ hydrolysis was markedly altered by PMA treatment while that mediated by G-protein remained unimpaired. Second, although short-term pretreatment with PMA resulted in inhibition of angiotensin II- and epinephrine-stimulated InsP formation, long-term pretreatment resulted in a markedly different change in responsiveness to these two agonists. That is, the InsP response to epinephrine was similar to that observed in control cells, but InsP accumulation in response to angiotensin II (and EGF and [Arg^8]vasopressin) were enhanced significantly over control responses. The reason for this selective sensitization of the phosphoinositide pathway for one receptor but not another is not clear. Perhaps activation of α_1-adrenergic receptors results in homologous receptor desensitization by more than one mechanism, *i.e.* one mechanism that is protein kinase C-dependent and one that does not require protein kinase C. If such were the case, then down-regulation of protein kinase C would not necessarily be expected to increase the InsP response to α_1-receptor stimulation.

The current data cannot completely rule out the possibility that phorbol esters sensitize the phosphoinositide signaling pathway by an action that occurs in parallel with down-regulation of protein kinase C. Within the sensitivity limits of the assay, long-term exposure of WB cells with both the lower, *i.e.* 600 nM, and the higher, *i.e.* 10 μM, concentrations of PMA caused similar 90–100% losses of protein kinase C activity. However, pretreatment with the higher concentration consistently elicited a more pronounced potentiation of hormone responsiveness. The reason for this phenomenon is not clear. It is conceivable that at high concentrations, PMA acts to impair other protein kinases or processes in addition to protein kinase C. Alternatively, it is possible that undetectably small amounts of active protein kinase C remain after chronic pretreatment with the lower but not the higher concentration of PMA. Thus we cannot rule out the possible contribution of a small residual amount of protein kinase C activity that imposes a negative regulation, albeit very modest, on the phosphoinositide signaling pathway. Cells pretreated with the higher concentration of phorbol ester presumably would be under no such negative regulation and thus capable of eliciting a relatively greater response to agonist stimulation. Further studies will be necessary to determine whether phorbol esters alter hormone responsiveness by modifying protein kinase C activity alone.

Agonist-induced desensitization is a property shared by a variety of cell surface receptors, and several molecular mechanisms apparently are involved in this process. Many hormone receptors undergo agonist-induced internalization as a mechanism for reducing the cells capacity to respond to subsequent hormonal stimulation. Recently, it has been shown that receptor phosphorylation by specific receptor kinases (50, 51) or cAMP-dependent protein kinase (52) may be important in regulation of receptor responsiveness to stimulation by agonist. Little is known about the mechanisms involved in agonist-induced desensitization of receptors linked to $PtdIns(4,5)P_2$ hydrolysis, although a regulatory role for protein kinase C in this process has been suggested by several studies. For example, protein kinase C-stimulated

phosphorylation of the α_1-adrenergic receptor has been shown to be correlated with a decrease in affinity for agonist binding and a marked attenuation in α_1-receptor agonist-stimulated turnover of phosphoinositides (53). Similarly, EGF stimulates PtdIns(4,5)P_2 hydrolysis (54–56) and Ca^{2+} mobilization (56–58) in A431 epidermoid carcinoma cells and short-term activation of protein kinase C by phorbol ester in these cells results in phosphorylation of the EGF receptor (59, 60) and loss of EGF-stimulated InsP formation (54) and Ca^{2+} mobilization (57, 58). In addition, phorbol esters have been shown to increase markedly the rate of internalization of the EGF receptor on KB human epidermoid carcinoma cells (61) and WB cells.[3] The present results are consistent with the idea that protein kinase C activation provides a negative feedback regulation of hormone-induced PtdIns(4,5)P_2 hydrolysis and Ins(1,4,5)P_3 production; angiotensin II receptor-mediated PtdIns(4,5)P_2 hydrolysis very rapidly undergoes agonist-induced desensitization, and this process can be prevented by long-term pretreatment with phorbol esters and a consequential down-regulation of protein kinase C. In support of these observations in WB cells, Brown and co-workers (20) very recently reported, and we have confirmed,[4] that bombesin-stimulated accumulation of inositol phosphates in Swiss 3T3 cells also is potentiated after long-term pretreatment with phorbol ester. Since DAG is also a product of PtdIns(4,5)P_2 hydrolysis and an endogenous activator of protein kinase C, then one result of hormone-induced increases in DAG levels and activation of protein kinase C may be the negative feedback regulation of the phosphoinositide signaling pathway to further agonist stimulation. WB cells should provide a useful model system for the further study of the role of protein kinase C in these processes.

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